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ORIGINAL ARTICLE

Evaluation of Lipotropic Effect of Herbal Formulation on Hepatic Fat Accumulation in Rats Fed with Methionine-Choline Deficient Diet

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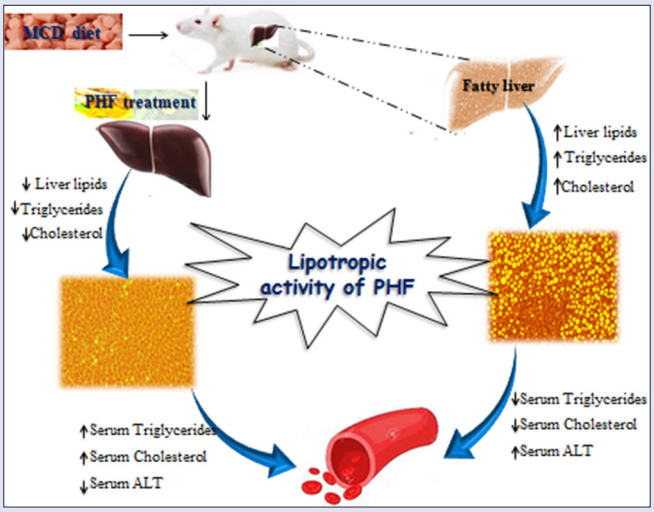
ABSTRACT

Background: Choline is an essential lipotropic nutrient for regulating fatty acid synthesis and hepatic lipid mobilization. Deficiency of choline causes fatty liver leading to dysregulated liver function. **Objective:** To investigate the lipotropic activity of the proprietary herbal formulation (PHF) containing *Acacia nilotica* and *Curcuma longa*. **Materials and Methods:** Fatty liver disease was induced in Wistar rats by feeding methionine/choline-deficient (MCD) diet for 4 weeks. Animals were concurrently treated with PHF (at 50, 100, 200, and 400 mg/kg rat body weight/day) for 4 weeks. Methionine/choline-sufficient (MCS) diet-fed rats were used as control. Serum biochemistry and liver parameters were determined at the end of experimental period. Further, anti-lipogenic and lipolytic activity of PHF extract was studied in HepG2 cells. **Results:** Rats fed with MCD diet, showed significant increase in liver lipids, triglycerides, cholesterol, thiobarbituric acid reactive substance, and serum alanine transaminase (ALT) and decreased serum triglyceride level compared to MCS diet-fed rats indicating significant fat accumulation and liver damage. PHF treatment significantly decreased the liver lipids, triglyceride and serum ALT compared to MCD diet-fed rat. Histological evaluation revealed the restoration of hepatic architecture after PHF treatment. In *in vitro* studies, the PHF extract decreased the oleic acid induced fat accumulation in HepG2 cells. **Conclusion:** The study demonstrated the lipotropic effect of PHF evident from decreased fat accumulation and antilipogenic activity. These data suggests that PHF could be a potential supplement for preventing fatty liver.

Key words: Choline, hepatosteatosis, lipogenesis, lipolysis, lipotrope

SUMMARY

- Albino Wistar rats fed with methionine-choline deficient diet induced nonalcoholic fatty liver
- Concurrent treatment with proprietary herbal formulation (PHF) for 4 weeks decreased liver lipids, triglycerides, and cholesterol in rats
- PHF decreased the hepatic lipid accumulation by exhibiting the lipotropic activity
- PHF extract exhibited antilipogenic activity against oleic acid-induced lipogenesis in HepG2 cells.



Abbreviations used: MCD: Methionine and choline deficient; MCS: Methionine and choline sufficient; PHF: Proprietary herbal formulation; OA: Oleic acid; TBARS: Thiobarbituric acid reactive substance.

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INTRODUCTION

Choline, classified as Vitamin B, is a major lipotropic nutrient essential for liver health. It actively participates in liver lipid metabolism thereby mitigates abnormal accumulation of fat in the liver.[1,2] Choline is also capable of inhibiting fatty acid synthesis by downregulating fatty acid synthase gene expression as well as attenuating its activity, which is crucial during lipogenesis.[3,4]

Choline deficiency causes abnormal lipid accumulation in the liver affecting its function. Liver is a visceral organ, which is capable of regenerating its damaged tissue thereby maintaining the metabolic homeostasis. However, excessive lipid accumulation in the liver causes delayed hepatocyte regeneration leading to irreversible liver diseases like steatohepatitis and liver cirrhosis. Hence, choline plays an important role in maintaining the hepatic lipid homeostasis. Choline is essential not only for humans,

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but also for animals and birds. Deficiency of choline in livestock animals compromises the liver function leading to decreased carcass yield, egg production and low quality milk with decreased fat content.[5,6] Therefore, choline is necessary in livestock to maintain their liver health and to increase the yield and quality traits of carcass and egg. Unfortunately, the availability of choline from regular diet is inadequate to the animals.[7] To overcome the inadequacy, synthetic choline is supplemented in the diet.

In practice, choline is supplemented as synthetic choline chloride. But it has a disadvantage of accelerating the carcinogen formation in gastrointestinal tract of animals and birds.[8,9] In ruminants, choline is rapidly and extensively degraded by rumen microbes before it reaches the intestine.[10] Furthermore, the choline chloride is very corrosive and requires special storage and handling equipment and is not suitable for inclusion in concentrated vitamin premix. To overcome these drawbacks, an attempt was made in search for an alternative to synthetic choline chloride.

Keeping in view, the lipotropic and hepatoprotective effect of the plant based food and traditional herbs,[11] a unique herbal lipotropic feed supplement was formulated. A proprietary herbal formulation (PHF) containing *Acacia nilotica* and *Curcuma longa* was developed and tested for its lipotropic activity. *A. nilotica* is demonstrated to prevent the hepatocellular damage induced by acetaminophen.[12,13] Furthermore, scientific evidences are available for the hepatoprotective activity of *C. longa*.[14,15] Based on its diversified pharmacological properties, an attempt was made to study the lipotropic effect of the PHF containing *A. nilotica* and *C. longa* in methionine and choline deficient (MCD) diet-induced fatty liver in rats. Methionine-Choline deficiency is a commonly used rodent model to induce hepatosteatosis and to study the lipotropic agents.[16,17] Further, the effect of PHF on intracellular lipid synthesis and lipolysis activity was investigated in human hepatocarcinoma cells (HepG2).

MATERIALS AND METHODS

In vivo study

Test substance

Kolin plus, a PHF is a blend of *A. nilotica* and *C. longa* optimized to contain not <8.0% polyphenols was developed by Natural Remedies Pvt. Ltd, Bangalore, India.[18]

Animals and diets

All the animal procedures were approved by Institutional Animal Ethics Committee of Natural Remedies Pvt. Ltd., Bangalore (IAEC/PCL/04/06.15). Animals were maintained in a controlled temperature (22°C ± 3°C), relative humidity (between 30% and 70%) and regular light cycle (12 h light, 12 h dark). Thirty-six male albino Wistar rats were divided into six groups with six animals per group. Group I and II to VI were fed with MCS and MCD diet respectively for 4 weeks. Group III, IV, V and VI were concurrently treated with PHF at 50, 100, 200 and 400 mg/kg body weight/day *p.o.,* respectively, after suspending PHF in 0.5% w/v carboxyl methyl solution (CMC). Group I and II were administered with CMC at 10 ml/kg body weight/day and were considered as positive and negative control respectively. MCD diet (A02082002B) and methionine and choline sufficient (MCS) diets (A02082003B) were obtained from Research diets Inc., USA. Ultraviolet treated water *ad libitum* was provided to rats.

After 4 weeks, body weight of each rat was recorded, blood was collected and animals were sacrificed. Liver and epididymal fat were excised and their weights were recorded.

Serum biochemistry

Serum triglyceride, alanine transaminase (ALT), and cholesterol levels were estimated using colorimetric assay kits (Arkray, Surat, India) as per manufacturer's protocol.

Liver parameters

Liver parameters include liver weight, liver lipids, triglycerides, cholesterol, thiobarbituric acid reactive substance (TBARS), and histological analysis.

Relative liver weight was computed for 100 g of rat body weight at terminal sacrifice.

The total lipid content in the liver tissue was determined by Folch method.[19] In brief, 1 g of liver tissue was homogenized with 20 mL of Chloroform–Methanol (2:1) mixture and centrifuged. The supernatant was mixed with 0.73% sodium chloride in the ratio of 5:1 and centrifuged. The lower organic phase consisting of lipids was transferred to a round bottom flask. After solvent evaporation, total lipid content was estimated by gravimetric method. The residue obtained was dissolved in 1 mL of isopropanol and analyzed for cholesterol and triglyceride levels using colorimetric assay kit (Arkray, Surat, India) as per manufacturer's protocol.

Liver TBARS was determined by Buege procedure, with minor modifications.[20] Liver tissue (500 mg) was homogenized in ice cold phosphate-buffered saline. Homogenate (100 µl) was added to trichloroacetic acid–thiobarbituric acid–hydrochloric acid reagent (200 µl) and incubated for 15 min in boiling water bath. The samples were then centrifuged and the supernatant (150 µl) was transferred to 96-well micro plate and absorbance was measured at 535 nm (Molecular devices, USA). The results are expressed as µM/g of liver tissue (Malondialdehyde equivalent).

A section of liver tissue was fixed in 10% neutral buffered formalin and the severity of histological changes was assessed after hematoxylin and eosin (H and E) staining. The histopathological analysis was performed by pathologist using the widely accepted Nonalcoholic Steatohepatitis Clinical Research Network histological scoring system for nonalcoholic fatty liver disease as described by Kleiner *et al*., 2005.[21] Briefly, semi quantitative scoring of steatosis was assigned as follows: Grade 0 = no fatty hepatocytes; Grade 1 = fatty hepatocytes occupying <33% of hepatic parenchyma; Grade 2 = 34%–66% of fatty hepatocytes; Grade 3 = occupying >66%. For ballooning; Grade 0 = No balloon cells; Grade 1 = few balloon cells; Grade 2 = prominent ballooning. Four fields were randomly selected from each section and the individual scores were assigned for each parameter. Inflammation was quantified by counting the inflammatory foci (group of ≥5 leukocytes) in 20 consecutive high power fields (HPF): Grade 0 = no inflammatory foci; Grade 1 = <2 inflammatory foci/HPF; Grade 2 = 2–4 foci/HPF; and Grade 3 = >4 inflammatory foci/HPF. The accumulation of fat in the liver was studied using oil red O staining.

Adipose cell size

Epididymal fat was fixed in 10% neutral buffered formalin and embedded in paraffin wax. After H and E staining of gonadal fat sections (3 µm thick), random pictures (6–10) were taken from different fields at ×20 with a DP2-BSW camera (Olympus Corporation, Japan). The area of each adipocyte was measured manually using Olympus DP2-BSW software, Japan. On average, 30 fat cells were measured from each section.

In vitro study

Preparation of proprietary herbal formulation extract

The blend of *A. nilotica* and *C. longa* (PHF) was refluxed with methanol in round bottom flask for 1.5 h at 65°C–70°C. The refluxed methanol was passed through 100 mesh filter cloth. The filtered extract was concentrated and dried under vacuum to obtain extract A with a percentage yield of 14.2% (W/W). The marc obtained after methanolic extraction was dried and refluxed thrice with water and the resulting

extract was concentrated to obtain extract B with a percentage yield of 5.3% (W/W). The PHF extract was made by mixing extracts A and B. This PHF extract was evaluated for its lipogenesis and lipolytic activity in HepG2 cells.

Lipogenesis and lipolysis

HepG2 cells were obtained from American Tissue Culture Collection, Rockville, MD, USA and cultured in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

In vitro anti-lipogenic assay was performed as per method described by Sung *et al.,* 2011.[22] Synthetic choline chloride was used as positive control. In brief, cells were plated at a density of 5×10^3 cells/well on a 96-well microplate in MEM supplemented with 10% FBS and incubated at 37°C for 24 h. After incubation, lipogenesis was induced by 2 mM Oleic acid (OA-dissolved in 0.1% dimethyl sulfoxide [DMSO]) supplemented with various concentration of PHF extract/synthetic choline chloride at 0–50 µg/mL of 0.1% DMSO and incubated for 48 h. Cells were washed with PBS and fixed in 10% formalin for 10 min. After fixation, cells were washed with 60% isopropanol and stained with oil red O dye (5 mg/mL isopropanol) for 15 min. Cells were then washed exhaustively with distilled water and the dye retained in the accumulated lipids was eluted with isopropanol and quantified by measuring the absorbance at 500 nm in microplate reader (Molecular Devices, USA) and compared with OA control group.

Lipolysis was assessed by quantifying the glycerol released into the medium, a marker of lipolysis. After 48 h of lipogenesis induced by 2 mM OA, overnight serum starved cells were incubated with various concentrations of PHF extract (0–50 µg/mL) for 4 h. Glycerol release was then quantified using colorimetric assay kit (Enzychrome, USA). Briefly, 10 µL of the medium was incubated with free glycerol reagent for 20 min and measured the absorbance at 570 nm and compared with OA control.

Statistical analysis

In vivo and *in vitro* raw data was processed using statistical software SPSS Version 21 (IBM Corp., New York, USA) and GraphPad Prism Version 5.01 (GraphPad software, Inc., CA, USA), respectively. *In vitro* data was pooled from three independent experiments with three replicates in each experiment. Statistical analysis was performed using one-way analysis of variance followed by *post hoc* Bonferroni test for homogenous data and Dunnet-T3 test for heterogeneous data. $P \leq 0.05$ was considered as statistically significant. Values are expressed as mean ± standard error of mean.

RESULTS

In vivo study

Effect of proprietary herbal formulation on serum biochemistry

The serum triglyceride and cholesterol level in MCD diet-fed rats was significantly low when compared to MCS diet-fed rats and the treatment with PHF at 200 mg/kg/day for 4 weeks showed significant increase in serum triglyceride level when compared to untreated rats fed with MCD diet. While PHF treatment increased the serum cholesterol levels nonsignificantly. MCD diet-fed negative control group exhibited a significant increase in serum ALT levels compared to MCS diet-fed rats demonstrating the hepatic damage. While treatment with PHF at 100, 200 and 400 mg/kg significantly decreased the ALT levels compared to MCD diet-fed rats [Table 1].

Effect of proprietary herbal formulation on liver parameters

MCD diet-fed rats showed a significant increase in relative liver weight compared to MCS diet-fed rats. Treatment with PHF at all dose levels significantly decreased the relative liver weight compared to MCD diet-fed rats [Table 2].

The liver total lipids, triglyceride and cholesterol levels were significantly increased in MCD diet-fed rats when compared to MCS diet-fed rats demonstrating the accumulation of lipids in the liver. Statistical analysis indicated that treatment with PHF at 100, 200 and 400 mg/kg significantly decreased the liver total lipids and triglycerides levels and nonsignificantly decreased the liver cholesterol levels when compared to MCD diet-fed rats [Table 2].

The oxidative damage to the liver was measured by TBARS. MCD diet-fed rats exhibited a significant increase in TBARS level while PHF treatment at 100, 200 and 400 mg/kg demonstrated a nonsignificant decrease in the TBARS level [Table 3].

The histological sections of liver were scored for steatosis, ballooning, and inflammation after H and E staining [Figure 1]. The score for steatosis, inflammation, and ballooning in MCD diet-fed group is 3.6, 1.55, and 3.55, respectively, whereas treatment with PHF significantly decreased the severity score for liver damage up to 0.95, 0.55, and 0.95, respectively [Figure 2]. Liver sections stained with oil red O stain revealed an increased fat accumulation in MCD diet-fed rats while treatment with PHF has decreased the fat accumulation [Figure 3].

Effect of proprietary herbal formulation on adipose cell size

The adipose cell size of MCD diet-fed group decreased by 58.45% compared to MCS diet-fed group [Figure 4] However, MCD diet-fed group treated with PHF concurrently (400 mg/kg) exhibited 94% increase in cell size compared to MCD group.

In vitro

Effect of proprietary herbal formulation extract on lipogenesis and lipolysis in HepG2 cells

The anti-lipogenic activity of PHF extract was evaluated in OA induced lipid synthesis in HepG2 cells. The quantitative analysis of oil red O staining revealed higher absorbance in the OA treated cells when compared to noninduced control. While treatment with PHF extract or synthetic choline chloride at 12.5, 25 and 50 µg/mL showed

Table 1: Effect of proprietary herbal formulation on serum triglycerides and cholesterol and ALT levels

Groups	ALT (IU/L)	Serum triglycerides (mg/dl)		Serum cholesterol (mg/dl)	
	Day 28	Day 0	Day 28	Day 0	Day 28
MCS (10 ml/kg: 0.5% CMC)	13.85±1.26	81.04±5.94	152.87±10.80	69.98±3.67	54.03±6.47
MCD (10 ml/kg: 0.5% CMC)	79.16±22.53*	77.29±6.46	21.13±3.02*	61.02±5.15	15.87±2.43
MCD + PHF (50 mg/kg/day)	36.17±9.48	92.54±11.28	38.16±8.59	64.05±5.59	28.94±7.00
MCD + PHF (100 mg/kg/day)	22.88±3.12#	83.26±9.01	40.54±6.86	61.57±2.57	35.73±5.04
MCD + PHF (200 mg/kg/day)	25.89±3.22#	90.45±4.36	36.00±1.12#	68.20±4.57	34.27±6.49
MCD + PHF (400 mg/kg/day)	25.90±1.95#	84.58±5.54	44.37±7.13	58.23±3.30	35.64±7.78

Values are expressed as mean±SEM; n=6. *P≤0.05, MCD diet group versus MCS diet group, #P≤0.05, treatment groups versus MCD diet groups. MCS: Methionine and choline sufficient; CMC: Carboxyl methyl solution; MCD: Methionine and choline deficient; PHF: Proprietary herbal formulation; SEM: Standard error of mean; ALT: Alanine transaminase

Table 2: Effect of proprietary herbal formulation on liver parameters

Groups	Relative organ weight (g)	Liver lipids (mg/g liver tissue)		
	Liver	Total lipids	Triglycerides	Cholesterol
MCS (10 ml/kg: 0.5% CMC)	2.71±0.11	43.80±3.49	4.54±1.31	1.22±0.20
MCD (10 ml/kg: 0.5% CMC)	4.87±0.09*	241.02±10.14*	67.67±1.96*	19.52±2.36*
MCD + PHF (50 mg/kg)	4.11±0.27#	158.41±27.93	33.09±7.36#	10.08±2.48
MCD + PHF (100 mg/kg)	3.45±0.16#	124.11±10.81#	29.44±3.91#	9.31±1.71
MCD + PHF (200 mg/kg)	3.69±0.13#	139.89±9.81#	37.56±1.79#	10.46±0.82
MCD + PHF (400 mg/kg)	3.55±0.13#	157.41±16.30#	45.81±2.28#	11.18±1.63

Values are expressed as mean±SEM; n=6. *P≤0.05, MCD diet group versus MCS diet group, #P≤0.05, treatment groups versus MCD diet groups. MCS: Methionine and choline sufficient; CMC: Carboxyl methyl solution; MCD: Methionine and choline deficient; PHF: Proprietary herbal formulation; SEM: Standard error of mean

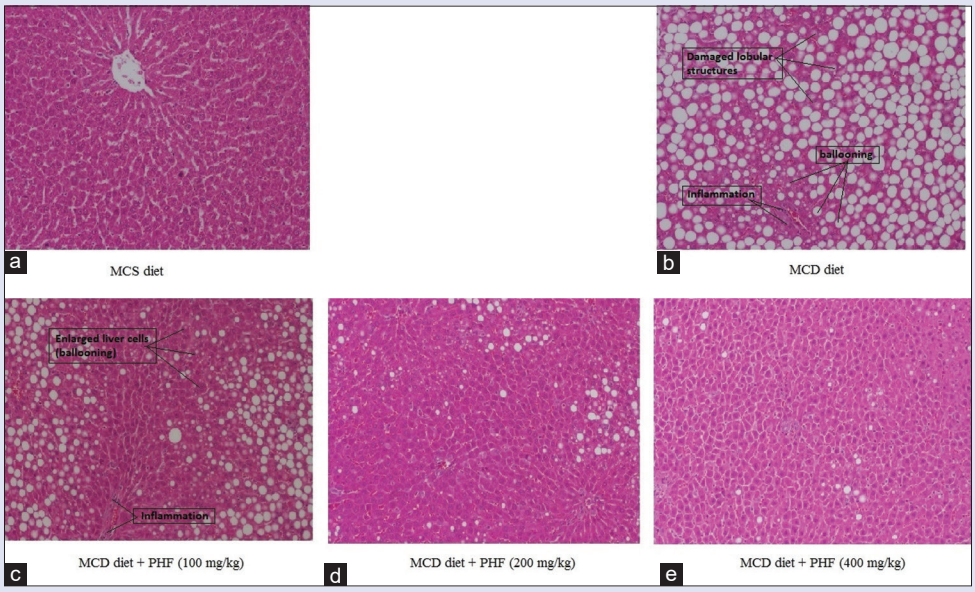

Figure 1: H and E staining of rat liver sections. Liver of rats fed a methionine- and choline-deficient/methionine- and choline-sufficient diet. (a) Methionine- and choline-sufficient diet showing normal architecture of the liver. (b) Methionine- and choline-deficient diet showing microvesicular steatosis, inflammation, and ballooning degeneration of hepatocytes. (c) Methionine and choline deficient + proprietary herbal formulation at 100 mg/kg. (d) Methionine and choline deficient + proprietary herbal formulation at 200 mg/kg. (e) Methionine and choline deficient + proprietary herbal formulation at 400 mg/kg showing a significant improvement in liver histology: Signs of steatosis, inflammation and ballooning are less pronounced compared to methionine and choline deficient diet (hematoxylin–eosin stain, at ×10)

a concentration dependent decrease in intracellular lipid accumulation compared to OA control [Figure 5].

The lipolytic activity of PHF extract and synthetic choline chloride was evaluated in OA induced lipogenesis in HepG2 cells. Treatment with PHF extract and synthetic choline chloride up to 50 µg/mL did not show increase in glycerol release when compared to OA control group [Figure 6].

DISCUSSION

Choline called a "lipotrope" is responsible for the export of hepatic lipids decreasing the abnormal accumulation of fat in the liver.[23,24] Furthermore, it potentiates the antioxidant capacity by participating in folate metabolism.[25] Although other lipotropes such as betaine, carnitine are involved in lipid mobilization, choline is considered a major lipotrope in all mammals including livestock.[26,27] However, endogenous synthesis of choline is inadequate to meet their functional needs and deficiency of choline leads to accumulation of lipid patches in hepatocytes, compromising the liver function.[11,28] Hence, choline is recommended to be supplemented in the diet. In practice, choline is supplemented to livestock as synthetic choline chloride. But it is highly hygroscopic and aggravates oxidative loss of vitamins.[8,9] These drawbacks results in poor bioavailability that eventually causes fatty liver and agricultural loss. This study aimed to demonstrate the lipotropic activity of the herbal formulation to maintain the liver health, consequently preventing the agricultural loss and low quality traits.[29]

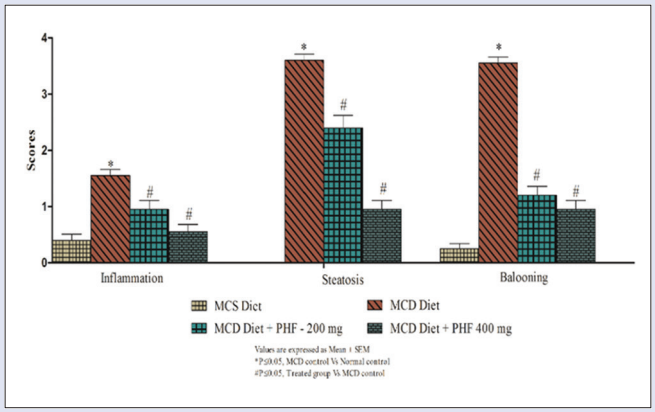

Figure 2: Histological scoring of H and E stained rat liver sections. Effect of proprietary herbal formulation on liver histology: Semiquantitative evaluation of liver – protection versus damage in methionine- and choline-deficient diet-fed rats (gradation/score) ×100

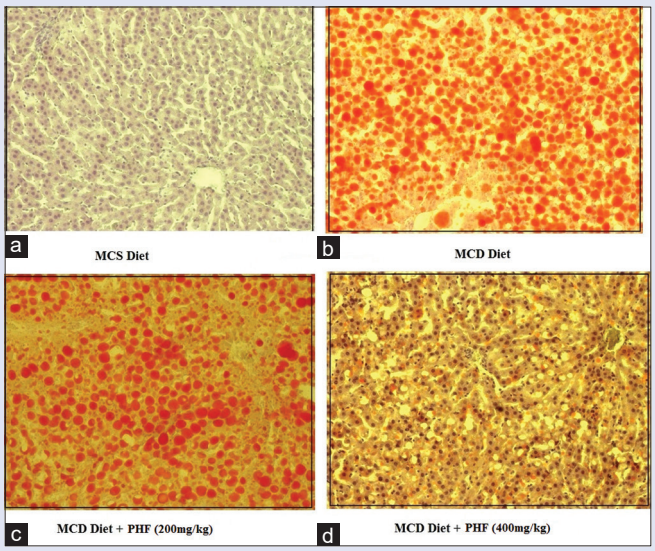

Figure 3: Oil red O staining of rat liver sections. Liver of rats fed a methionine- and choline-deficient/methionine- and choline-sufficient diet. (a) Methionine- and choline-sufficient diet showing normal architecture of the liver. (b) Methionine- and choline-deficient diet showing the fat accumulation. (c) Methionine and choline deficient + proprietary herbal formulation at 200 mg/kg. (d) Methionine and choline deficient + proprietary herbal formulation at 400 mg/kg showing a reduction in fat accumulation compared to methionine and choline deficient diet (Oil Red O stain, magnifications at ×10)

Table 3: Effect of proprietary herbal formulation on liver antioxidant

Groups	TBARS (μM)/g liver tissue
MCS (10 ml/kg: 0.5% CMC)	7.98±0.61
MCD (10 ml/kg: 0.5% CMC)	60.74±12.99*
MCD + PHF (50 mg/kg)	66.00±11.53
MCD + PHF (100 mg/kg)	43.60±7.31
MCD + PHF (200 mg/kg)	52.49±7.80
MCD + PHF (400 mg/kg)	38.45±5.12

Values are expressed as mean±SEM; n=6. *P≤0.05, MCD diet group versus MCS diet group. MCS: Methionine and choline sufficient; CMC: Carboxyl methyl solution; MCD: Methionine and choline deficient; PHF: Proprietary herbal formulation; TBARS: Thiobarbituric acid reactive substance; SEM: Standard error of mean

Evidence supports that polyphenol rich diet aids in the prevention and treatment of fatty liver as that of choline by increasing the hepatic lipid export.[30,31] In view of the lipid mobilizing effects of herbs that will substitute the lipotropic activity of choline, a novel PHF containing *A. nilotica* and *C. longa* standardized to contain not <8% polyphenols was developed. The present study was conducted to evaluate the lipotropic activity of PHF in MCD diet-fed rats. In addition, the anti-lipogenic and lipolytic activities were evaluated and compared with synthetic choline chloride in *in vitro* HepG2 cells.

Choline and methionine deficient diet are widely used to induce hepatosteatosis and to study the lipotropic effect in animals. MCD diet induces lipid accumulation by increasing the fatty acid uptake and triglyceride synthesis; and by decreasing the β-oxidation or hepatic secretion of very low density lipoproteins that eventually leads to liver injury.[32] These effects decreases the degree of mobilization of stored lipid from liver to the adipose tissue affecting the liver function and metabolic pathways.[33] To induce a prominent liver damage, methionine, a precursor for choline synthesis, was also withdrawn from the diet.[34]

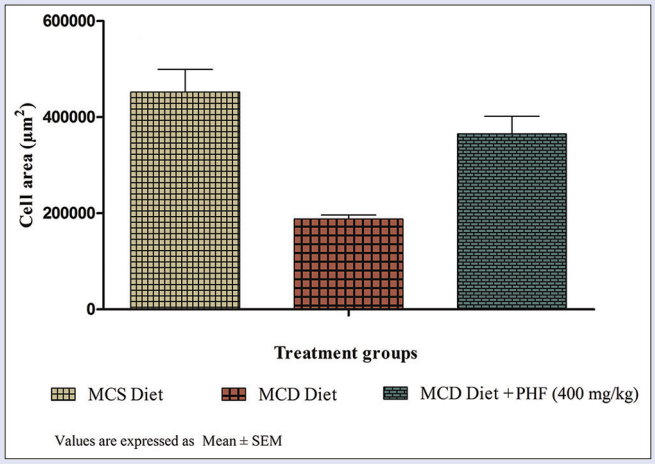

Figure 4: Adipose cell area. Adipose cell area measured after H and E staining of adipose tissue indicated a nonsignificant decrease in methionine- and choline-deficient diet-fed group, while proprietary herbal formulation treated group has shown a nonsignificant increase in cell area compared to methionine- and choline-deficient diet-fed group

MCD diet decreased the serum triglycerides and cholesterol levels compared to MCS diet-fed rats, whereas treatment with PHF increased the serum triglycerides, cholesterol, compared to MCD diet-fed negative control group. Serum ALT is a reliable and sensitive marker of liver health.[35] Excess fat accumulation in the liver causes hepatocellular damage, releasing the hepatic enzymes into the circulation. Increased serum ALT levels in MCD diet-fed group confirmed the hepatic injury in rats, whereas treatment with PHF, decreased the ALT levels by 2.5 fold compared to MCD diet-fed group demonstrating the hepatoprotective activity.

Abnormal accumulation of lipids in the liver is a hallmark of hepatosteatosis.[36] Increased hepatic lipids, decreased serum triglycerides and cholesterol in MCD diet-fed group confirmed the state of hepatosteatosis. A substance is considered as a lipotrope when it increases the hepatic fat export which is indicated by decreased hepatic lipid content and increased serum or adipose lipids. PHF treatment exhibited lipotropic activity by decreasing the hepatic lipids. High energy diet induces oxidative DNA damage by increasing the production of reactive oxygen species (ROS).[37] ROS attacks on polyunsaturated fatty acid results in lipid peroxidation, producing TBARS which is measured in terms of malondialdehyde.[38] In the present study, PHF treatment nonsignificantly showed a decreased trend for lipid peroxidation/damage and consequently the hepatic oxidative damage induced by MCD diet.

Steatosis is characterized by accumulation of lipids and inflammation in the liver leading to oxidative stress.[39] Histological examination of liver is a promising tool to understand the severity of hepatosteatosis.[40] The H and E stained liver sections of MCD diet-fed group revealed significant steatosis with damaged lobular structures, enlarged liver cells (ballooning) and inflammation. Unlike MCD diet-fed group, hepatocyte of PHF intervention groups did not differ significantly from the MCS diet-fed control group. The restoration of hepatic architecture by PHF treatment was also observed through oil red O staining. The histological finding of steatosis corroborates the estimated hepatic total lipids.

It has been observed that adipose tissue dysfunction is in tight correlation with the progression of hepatosteatosis.[41] Similarly, our study confirmed the decrease in adipose cell size in MCD diet-fed rats which upon treatment with PHF has increased the cell size suggesting the redistribution of lipids from liver to adipose tissue, thereby maintaining the lipid homeostasis.

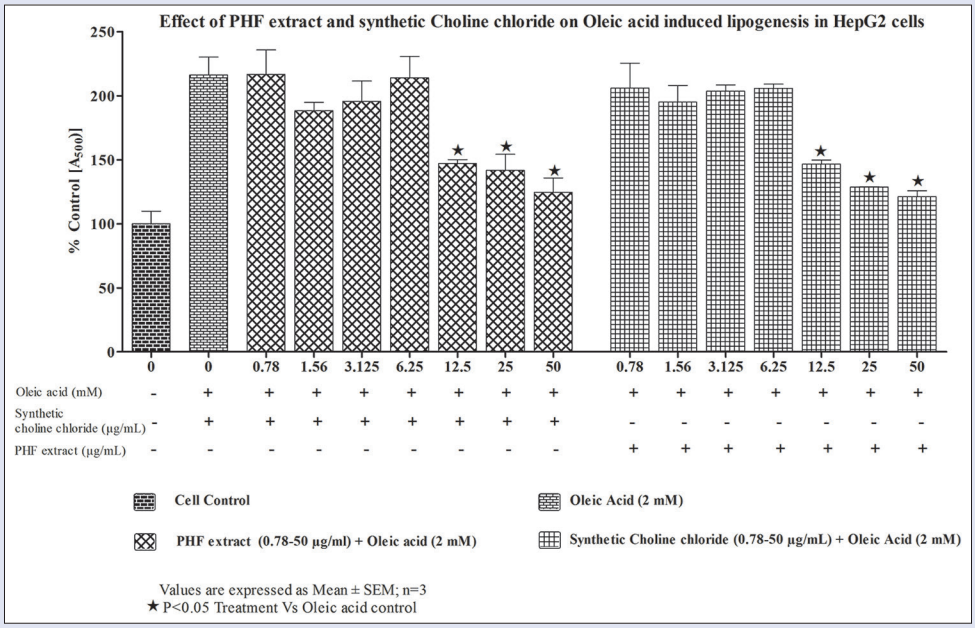

Figure 5: Effect of proprietary herbal formulation extract and synthetic choline chloride on Oleic acid induced lipogenesis in HepG2 cells. Effect of various concentrations of proprietary herbal formulation extract/synthetic choline chloride on oleic acid-induced lipogenesis in HepG2 cells. Lipogenesis was quantified by oil red O-based colorimetric assay and values are expressed as mean ± standard error of mean. "*" indicate significant different from the oleic acid treated group at the $P \leq 0.05$ levels

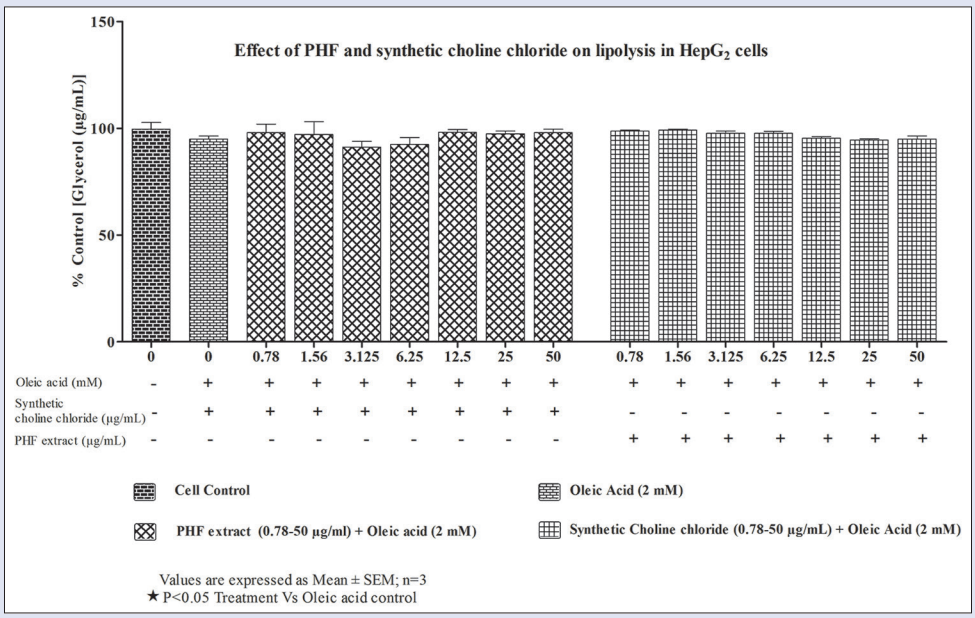

Figure 6: Effect of proprietary herbal formulation extract and synthetic choline chloride on lipolysis in HepG2 cells. Effect of various concentrations of proprietary herbal formulation extract/synthetic choline chloride on lipolysis in HepG2 cells. Lipolytic activity was measured by quantifying the amount of glycerol released into the medium and the values are expressed as mean ± standard error of mean

Apart from *in vivo* lipotropic activity of PHF, the other possible mechanism by which PHF decreases the hepatic lipid accumulation was studied using OA induced lipogenesis in HepG2 cells. Oil Red O staining demonstrated that OA stimulated hepatocytes had increased lipid accumulation compared with cell control. However, treatment with PHF extract or synthetic choline chloride decreased the lipid accumulation on OA induced lipogenesis signifying its anti-lipogenic activity. The anti-lipogenic activity of PHF appears to be similar to that of synthetic choline chloride. Effect of PHF to lyse the accumulated lipids induced by OA was also studied in HepG2 cells. During the process of lipolysis, the triglycerides get hydrolyzed and release the glycerol and free fatty acids. This glycerol release is measured after oil red O staining to determine the lipolytic activity.[42] PHF extract and synthetic choline chloride did not differ in glycerol release when compared to differentiated control. This suggests that choline does not possess lipolytic activity which is in correlation with the previous

study conducted in ob/ob obese mice.[43] However, PHF could prevent fatty liver by inhibiting lipid synthesis. By these means, it indicates that alike choline, PHF prevents the hepatic fat accumulation by exhibiting lipotropic and antilipogenic activity.

CONCLUSION

The present study demonstrated that PHF exhibit potent lipotropic activity in MCD diet induced hepatosteatosis in rats. In addition, PHF treatment inhibited OA-induced lipogenesis similar to synthetic choline chloride in HepG2 cells. Thus, the study findings suggest that PHF proves to be a promising candidate for the prevention of fatty liver. PHF could also be proven beneficial to the agricultural livestock in maintaining their liver health. However, further studies in the target species are warranted.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Farina G, Kessler AD, Ebling PD, Marx FR, César R, Ribeiro AM. Performance of broilers fed different dietary choline sources and levels. Cienc Anim Bras 2017;18:e37633.
2. Mehedint MG, Zeisel SH. Choline's role in maintaining liver function: New evidence for epigenetic mechanisms. Curr Opin Clin Nutr Metab Care 2013;16:339-45.
3. Sherriff JL, O'Sullivan TA, Properzi C, Oddo JL, Adams LA. Choline, its potential role in nonalcoholic fatty liver disease, and the case for human and bacterial genes. Adv Nutr 2016;7:5-13.
4. Zhu J, Wu Y, Tang Q, Leng Y, Cai W. The effects of choline on hepatic lipid metabolism, mitochondrial function and antioxidative status in human hepatic C3A cells exposed to excessive energy substrates. Nutrients 2014;6:2552-71.
5. Wen ZG, Tang J, Hou SS, Guo YM, Huang W, Xie M. Choline requirements of white Pekin ducks from hatch to 21 days of age. Poult Sci 2014;93:3091-6.
6. Sharma BK, Erdman RA. Effects of dietary and abomasally infused choline on milk production responses of lactating dairy cows. J Nutr 1989;119:248-54.
7. Emmert JL, Baker DH. A chick bioassay approach for determining the bioavailable choline concentration in normal and overheated soybean meal, canola meal and peanut meal. J Nutr 1997;127:745-52.
8. Calderano AA, Nunes RV, Rodrigueiro RJ, César RA. Replacement of choline chloride by a vegetal source of choline in diets for broilers. Cienc Anim Bras 2015;16:37-44.
9. Zeisel SH, daCosta KA, Youssef M, Hensey S. Conversion of dietary choline to trimethylamine and dimethylamine in rats: Dose-response relationship. J Nutr 1989;119:800-4.
10. Jayaprakash G, Sathiyabarathi M, Robert MA, Tamilmani T. Rumen-protected choline: A significance effect on dairy cattle nutrition. Vet World 2016;9:837-41.
11. Narayanankutty A, Palliyil DM, Kuruvilla K, Raghavamenon AC. Virgin coconut oil reverses hepatic steatosis by restoring redox homeostasis and lipid metabolism in male wistar rats. J Sci Food Agric 2018;98:1757-64.
12. Verma P, Singh SP, Vind SK, Kumar GR, Rao CV. Protective Effect of *acacia nilotica* (Bark) against anti-tuberculardrug induced hepatic damage an experimental study. Int J Pharm Pharm Sci 2014;6:75-9.
13. Kannan N, Sakthivel KM, Guruvayoorappan C. Protective effect of *Acacia nilotica* (L.) against acetaminophen-induced hepatocellular damage in wistar rats. Adv Pharmacol Sci 2013;2013:987692.
14. Nwozo SO, Osunmadewa DA, Oyinloye BE. Anti-fatty liver effects of oils from *Zingiber officinale* and *curcuma longa* on ethanol-induced fatty liver in rats. J Integr Med 2014;12:59-65.
15. Yiu WF, Kwan PL, Wong CY, Kam TS, Chiu SM, Chan SW, *et al.* Attenuation of fatty liver and prevention of hypercholesterolemia by extract of *curcuma longa* through regulating the expression of CYP7A1, LDL-receptor, HO-1, and HMG-coA reductase. J Food Sci 2011;76:H80-9.
16. Jha P, Claudel T, Baghdasaryan A, Mueller M, Halilbasic E, Das SK, *et al.* Role of adipose triglyceride lipase (PNPLA2) in protection from hepatic inflammation in mouse models of steatohepatitis and endotoxemia. Hepatology 2014;59:858-69.
17. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, *et al.* Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482:179-85.
18. Srinivasa Rao B, Chandrasekaran CV, Srikanth HS, Sasikumar M, Edwin Jothie R, Haseena B, *et al.* Mutagenicity and acute oral toxicity test for herbal poultry feed supplements. J Toxicol 2018;2018.
19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
20. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302-10.
21. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-21.
22. Sung JH, Chon JW, Lee MA, Park JK, Woo JT, Park YK. The anti-obesity effect of lethariella cladonioides in 3T3-L1 cells and obese mice. Nutr Res Pract 2011;5:503-10.
23. Choi YI, Ahn HJ, Lee BK, Oh ST, An BK, Kang CW. Nutritional and hormonal induction of fatty liver syndrome and effects of dietary lipotropic factors in egg-type male chicks. Asian-Australas J Anim Sci 2012;25:1145-52.
24. Overton TR, Waldron MR. Nutritional Management of Transition Dairy Cows: Strategies to Optimize Metabolic Health. J. Dairy Sci 2004;87:E105-19.
25. Drews K. Folate metabolism – Epigenetic role of choline and vitamin B12 during pregnancy. Ginekol Pol 2015;86:940-6.
26. McHenry EM, Patterson JM. Lipotropic factors. Physiol Rev 1944;24:128-67.
27. Erdman RA, Shaver RD, Vandersall JH. Dietary choline for the lactating cow: Possible effects on milk fat synthesis. J Dairy Sci 1984;67:410-5.
28. Mullen GP, Mathews EA, Vu MH, Hunter JW, Frisby DL, Duke A, *et al.* Choline transport and *de novo* choline synthesis support acetylcholine biosynthesis in caenorhabditis elegans cholinergic neurons. Genetics 2007;177:195-204.
29. Crespo R, Shivaprasad HL. Developmental, metabolic and other noninfectious disorders, In: Swayne DE, editor, Diseases of poultry. New Jersy: John Wiley and Sons, Inc; 2013. p. 1233-70.
30. Rodriguez-Ramiro I, Vauzour D, Minihane AM. Polyphenols and non-alcoholic fatty liver disease: Impact and mechanisms. Proc Nutr Soc 2016;75:47-60.
31. Gangane GR, Gaikwad NJ, Ravikanthand K, Maini S. The Comparative effects of synthetic choline and herbal choline on hepatic lipid metabolism in broilers. Vet World 2010;3:318-20.
32. Park HS, Jeon BH, Woo SH, Leem J, Jang JE, Cho MS, *et al.* Time-dependent changes in lipid metabolism in mice with methionine choline deficiency-induced fatty liver disease. Mol Cells 2011;32:571-7.
33. Jung YA, Choi YK, Jung GS, Seo HY, Kim HS, Jang BK, *et al.* Sitagliptin attenuates methionine/choline-deficient diet-induced steatohepatitis. Diabetes Res Clin Pract 2014;105:47-57.
34. Chandler TL, White HM. Choline and methionine differentially alter methyl carbon metabolism in bovine neonatal hepatocytes. PLoS One 2017;12:e0171080.
35. Cui W, Chen SL, Hu KQ. Quantification and mechanisms of oleic acid-induced steatosis in hepG2 cells. Am J Transl Res 2010;2:95-104.
36. Fon Tacer K, Rozman D. Nonalcoholic fatty liver disease: Focus on lipoprotein and lipid deregulation. J Lipids 2011;2011:783976.
37. Higgs MR, Chouteau P, Lerat H. 'Liver let die': Oxidative DNA damage and hepatotropic viruses. J Gen Virol 2014;95:991-1004.
38. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81-128.
39. Chae MK, Park SG, Song SO, Kang ES, Cha BS, Lee HC, *et al.* Pentoxifylline attenuates methionine- and choline-deficient-diet-induced steatohepatitis by suppressing TNF-α expression and endoplasmic reticulum stress. Exp Diabetes Res 2012;2012:762565.
40. Levene AP, Kudo H, Armstrong MJ, Thursz MR, Gedroyc WM, Anstee QM, *et al.* Quantifying hepatic steatosis – more than meets the eye. Histopathology 2012;60:971-81.
41. Duval C, Thissen U, Keshtkar S, Accart B, Stienstra R, Boekschoten MV, *et al.* Adipose tissue dysfunction signals progression of hepatic steatosis towards nonalcoholic steatohepatitis in C57BL/6 mice. Diabetes 2010;59:3181-91.
42. Moussalli C, Downs RW, May JM. Potentiation by glucose of lipolytic responsiveness of human adipocytes. Diabetes 1986;35:759-63.
43. Wu G, Zhang L, Li T, Lopaschuk G, Vance DE, Jacobs RL. Choline deficiency attenuates body weight gain and improves glucose tolerance in ob/ob mice. J Obes 2012;2012:319172.